JOURNAL OF BACTERIOLOGY, Sept. 1999, p. 5600–5605 Vol. 181, No. 18
0021-9193/99/$04.00+0

Insertional Inactivation of Methylmalonyl Coenzyme A (CoA) Mutase and Isobutyryl-CoA Mutase Genes in *Streptomyces cinnamonensis*: Influence on Polyketide Antibiotic Biosynthesis

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The coenzyme B_{12}-dependent isobutyryl coenzyme A (CoA) mutase (ICM) and methylmalonyl-CoA mutase (MCM) catalyze the isomerization of *n*-butyryl-CoA to isobutyryl-CoA and of methylmalonyl-CoA to succinyl-CoA, respectively. The influence that both mutases have on the conversion of *n*- and isobutyryl-CoA to methylmalonyl-CoA and the use of the latter in polyketide biosynthesis have been investigated with the polyether antibiotic (monensin) producer *Streptomyces cinnamonensis*. Mutants prepared by inserting a hygromycin resistance gene (*hygB*) into either *icmA* or *mutB*, encoding the large subunits of ICM and MCM, respectively, have been characterized. The *icmA*::*hygB* mutant was unable to grow on valine or isobutyrate as the sole carbon source but grew normally on butyrate, indicating a key role for ICM in valine and isobutyrate metabolism in minimal medium. The *mutB*::*hygB* mutant was unable to grow on propionate and grew only weakly on butyrate and isobutyrate as sole carbon sources. ^{13}C-labeling experiments show that in both mutants butyrate and acetoacetate may be incorporated into the propionate units in monensin A without cleavage to acetate units. Hence, *n*-butyryl-CoA may be converted into methylmalonyl-CoA through a carbon skeleton rearrangement for which neither ICM nor MCM alone is essential.

Streptomycetes produce a wide variety of commercially important polyketide secondary metabolites, including the well-known macrolide and polyether antibiotics. The assembly of these antibiotics, catalyzed by the large family of polyketide synthases, requires malonyl coenzyme A (CoA), methylmalonyl-CoA, and ethylmalonyl-CoA as extender units, which are the precursors of the acetate-, propionate-, and butyrate-derived units, respectively, in the polyketide backbone (8, 11, 12).

Fatty acid and amino acid catabolisms are known to play an important role in supplying building blocks for polyketide biosynthesis. *n*-Butyryl-CoA and isobutyryl-CoA are known intermediates in fatty acid and valine catabolism, and both may be converted intact into methylmalonyl-CoA and ethylmalonyl-CoA in streptomycetes (Fig. 1). This is evidenced by the efficient incorporation of ^{13}C-labeled *n*- and isobutyrate into both the propionate- and butyrate-derived units in several polyketide antibiotics, including monensin A (20, 22), tylosin (18), and narasin (6). The coenzyme B_{12}-dependent isobutyryl-CoA mutase (ICM) appears to play a key role in these processes, by catalyzing the interconversion of *n*- and isobutyryl-CoA. However, the preferred route(s) for the conversion of *n*- and isobutyryl-CoA into methylmalonyl-CoA is less clearly defined. One possibility, consistent with results of ^{13}C-labeling studies, is the direct oxidation of isobutyryl-CoA, in several enzymatic steps, perhaps via methacrylyl-CoA and 3-hydroxybutyryl-CoA, to give methylmalonyl-CoA (path A in Fig. 1), although this route is different from the well-known pathway of valine catabolism in pseudomonads and mammals that leads via methylmalonic acid semialdehyde to propionyl-CoA (10, 15, 32) (Fig. 1). Another possibility is that *n*-butyryl-CoA might be oxidized at C-4 (ω oxidation) over several steps without fragmentation, to give succinyl-CoA, which may then be converted to methylmalonyl-CoA by methylmalonyl-CoA mutase (MCM) (path B in Fig. 1). Known routes to methylmalonyl-CoA presently include the carboxylation of propionyl-CoA, catalyzed by propionyl-CoA carboxylase, and the isomerization of succinyl-CoA, catalyzed by the coenzyme B_{12}-dependent MCM. Ethylmalonyl-CoA appears to be derived solely by carboxylation of *n*-butyryl-CoA.

To further explore the roles of MCM and ICM in the production of methylmalonyl-CoA, we report here the properties of *Streptomyces cinnamonensis* mutants in which either MCM or ICM has been inactivated by gene disruption. The genes encoding both enzymes have been cloned and sequenced from *S. cinnamonensis*, the producer of the polyether antibiotic monensin A (Fig. 1) (2, 33). MCM consists of two subunits, MutA and MutB, of 65 and 79 kDa, respectively, which show high sequence similarities to MCMs from other bacteria and mammals. So far, ICM has been isolated only from *S. cinnamonensis*, where it was found to comprise a large subunit of 65 kDa (IcmA) and a small subunit of ca. 17 kDa (IcmB) (33). The cloning and insertional inactivation of *icmA* from *S. cinnamonensis* were reported earlier (33). In this work, a mutant was prepared by inserting a hygromycin resistance gene (*hygB*) into *mutB*. The growth characteristics of both *icmA*::*hygB* and *mutB*::*hygB* mutants and the pattern of incorporation of ^{13}C-labeled precursors into the polyketide monensin A in each are reported below. The results indicate a key role for ICM in valine and isobutyrate metabolism under normal growth conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and fermentations. The bacterial strains and plasmids used in this study are listed in Table 1. *S. cinnamonensis* A3823.5, a high-yield producer of monensin A, was a gift of Eli Lilly & Co. (Indianapolis, Ind.). Phenotypic analysis of *S. cinnamonensis* strains was performed on solid minimal medium (9) with ammonium sulfate as nitrogen source and various carbon sources (50 mM). The growth was monitored after 7 to 10 days at 30°C.

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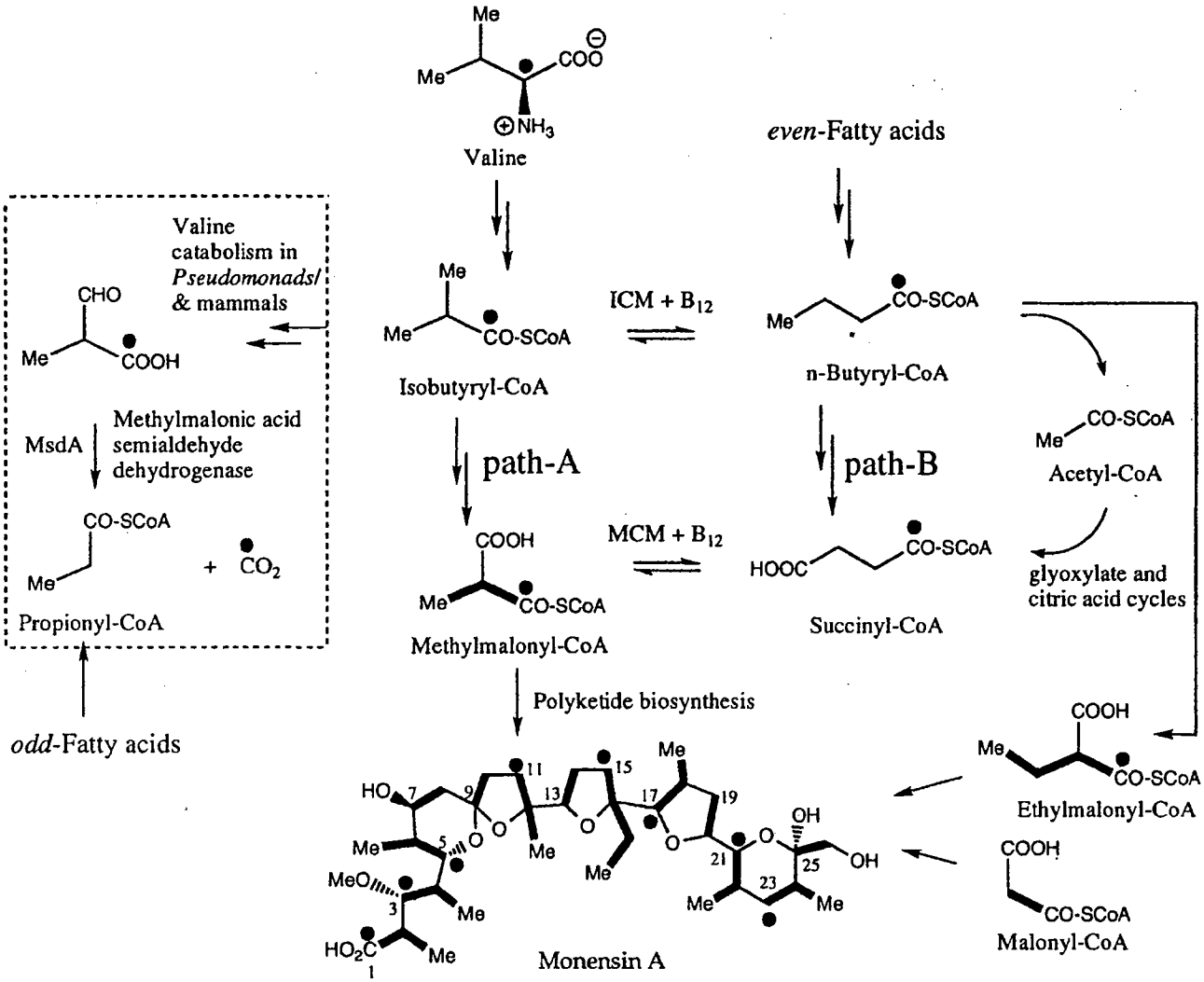

FIG. 1. Pathways of valine catabolism and the reactions catalyzed by MCM and ICM.

Chemicals. Chemicals were purchased from Fluka, Buchs, Switzerland. Sodium [1-^{13}C]butyrate and ethyl [1,3-$^{13}C_2$]acetoacetate were from Cambridge Isotope Laboratories, Inc. Sodium [1-^{13}C]isobutyrate was prepared as described earlier (22).

Insertional inactivation of *mutB*. In order to disrupt *mutB* in *S. cinnamonensis*, a 2.55-kb *Bam*HI fragment (2) containing almost the entire *mutB* gene was cloned into the *Bam*HI site of pUC18 to generate pOCI403. Subsequently, a hygromycin resistance gene (*hygB*) was cloned as a *Bam*HI/*Bgl*II fragment into the unique *Bgl*II site of pOCI403 to generate pOCI352. The *Bam*HI fragment of pOCI352 was isolated and cloned into the unique *Bam*HI cloning site of pGM160. The resulting plasmid, pOCI353, after passage through *Escherichia coli* ET12567 and *Streptomyces lividans* TK64, was used to transform *S. cinnamonensis* A3823.5. Selection for the disrupted *mutB* gene was performed as previously described (33).

Incorporation of ^{13}C-labeled precursors into monensin A. Singly labeled precursors (100 mg per culture) were fed batchwise in equal portions after 24, 36, and 48 h to two 60-ml cultures in a complex liquid medium (22, 28). Subsequently, monensin A was purified from the culture broth as in earlier work (22) and examined by inverse-gated $^{13}C\{^1H\}$ nuclear magnetic resonance (NMR) spectroscopy (20-s relaxation delay, 30° pulse) on a Bruker AMX600 spectrometer. Enrichment levels were determined by signal integration. The ethyl [1,3-$^{13}C_2$]acetoacetate (100 mg) was mixed with unlabeled ethyl acetoacetate (200 mg) and then batch fed to two liquid cultures (150 mg per culture) in three equal portions after 48, 60, and 72 h of growth. Typically, 30 to 70 mg of pure monensin A was obtained from each experiment.

RESULTS

Insertional inactivation of *S. cinnamonensis mutB*. Disruption of *mutB* was achieved by cloning a hygromycin resistance gene, *hygB*, into the unique *Bgl*II site of *mutB* (2). A restriction fragment containing the disrupted *mutB* and *hygB* genes was then cloned into pGM160, which carries also the thiostrepton resistance gene. The resulting plasmid, pOCI353, was used to transform protoplasts of *S. cinnamonensis* A3823.5. Isolation of the desired mutant was performed by selecting for Ts^s and Hg^r colonies at 39°C, as in earlier work (33). A Southern blot hybridization analysis with total DNA isolated from one clone (*mutB*::*hygB*) confirmed that the *mutB* gene had been disrupted, consistent with a double-crossover event (data not shown). The *mutB*::*hygB* mutant was devoid of MCM activity in cell extracts.

Phenotype analysis of the *S. cinnamonensis* mutant strains. The growth characteristics of wild-type (wt) *S. cinnamonensis* and the *mutB*::*hygB* and *icmA*::*hygB* mutants on solid minimal medium containing a single carbon source were determined, and the results are summarized in Table 2. When grown in a

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Streptomyces spp.		
S. cinnamonensis A3823.5	Monensin-overproducing strain	Eli Lilly & Co.
S. lividans TK64	*pro-2 str-6*	9
E. coli ET12567	*hsdS dam-3 dcm-6*	14
Plasmids		
pUC18		17
pGM160	Temperature-sensitive plasmid	16
pIJ963	Contains the *hygB* gene	13
pOCI403	Contains 2.3-kb *Bam*HI fragment of *mutB* in pUC18	This work
pOCI352	Hygromycin resistance gene cloned as *Bam*HI-*Bgl*II fragment in the *Bgl*II site of pOCI403	This work
pOCI353	Contains the disrupted *mutB* gene cloned as *Bam*HI fragment in pGM160	This work

complex oil-based medium (medium 5, described in reference 28), the wt and the two mutants grew equally well and produced comparable levels of monensin A. However, during growth in this medium addition of isobutyrate or valine at a ca. 5 mM concentration to the *icmA*::*hygB* mutant caused cessation of further growth and monensin production. In a chemically defined medium (4) containing mainly glucose, tyrosine, and valine as C and N sources, the growth of the *mutB*::*hygB* mutant was comparable to that of the wt, whereas the growth of the *icmA*::*hygB* mutant was weak.

Incorporation of ^{13}C-labeled precursors into monensin A. The ability of *n*-butyrate and isobutyrate to serve as precursors for monensin A biosynthesis in cultures of the mutant strains *mutB*::*hygB* and *icmA*::*hygB* was tested by adding *n*-[1-^{13}C]butyrate and [1-^{13}C]isobutyrate to shake cultures of each, as well as to wt *S. cinnamonensis*. Monensin A was subsequently isolated and examined by ^{13}C{^{1}H} NMR spectroscopy. The observed enrichments are summarized in Table 3. Only trace amounts of monensin A could be isolated from the *icmA*::*hygB* strain when it was fed with [1-^{13}C]isobutyrate. The addition of isobutyrate (or valine) to this mutant strongly inhibited further growth and monensin production. The same effect was not observed upon feeding the mutant with *n*-[1-^{13}C]butyrate. The *mutB*::*hygB* strain showed relatively high incorporations of ^{13}C in the propionate and butyrate units of monensin A upon being fed with both labeled butyrate and isobutyrate (Table 3).

Ethyl [1,3-$^{13}C_2$]acetoacetate was fed to the wt and both *S. cinnamonensis* mutants, the monensin A was subsequently isolated from each culture, and ^{13}C{^{1}H} NMR spectra were recorded. For monensin A from both mutants, highly enriched doublet signals were observed surrounding the natural abundance singlets for all positions derived from C-1 and C-2 of a propionate building block, i.e., in monensin A, C-1–C-2, C-3–C-4, C-5–C-6, C-11–C-12, C-17–C-18, C-21–C-22, and C-23–C-24 (see Fig. 2 for examples). This implies the formation of [1,2-$^{13}C_2$]methylmalonyl-CoA from [1,3-$^{13}C_2$]acetoacetyl-CoA in vivo. Also, the signals for C-15 and C-32, corresponding to the C-1 and C-3 positions in the butyrate building block, were strongly enriched (Fig. 2).

TABLE 2. Growth of *S. cinnamonensis* strains on solid minimal medium with various added carbon sources[a]

Carbon Source	wt	*mutB*::*hygB*	*icmA*::*hygB*
Glucose	+++	+++	+++
Succinate	+++	+++	+++
Valine	++	++	−
Acetate	++	++	++
Propionate	+++	−	+++
Butyrate	+++	+	+++
Valerate	+++	−	+++
Caproate	++	+	++
Isobutyrate	++	+	−
Isocaproic acid	++	+	−
Crotonic acid	++	+	++
Ethyl acetoacetate	++	+	++
rac-3-Hydroxybutyrate	+++	++	+++
None	−	−	−

[a] +++, strong, rapid, dense mycelial growth; ++, intermediate growth; +, slow, weak, but still clear growth; −, almost no mycelial growth, corresponding to growth without a carbon source.

DISCUSSION

ICM is an MCM-like enzyme from *S. cinnamonensis* comprising a large subunit (IcmA) of 566 residues (33) and a small

TABLE 3. Enrichments observed in monensin A after incorporation of *n*-[1-^{13}C]butyrate and [1-^{13}C]isobutyrate in the wt and in *mutB*::*hygB* and *icmA*::*hygB* mutants of *S. cinnamonensis*

Monensin carbon	*n*-[1-^{13}C]butyrate			[1-^{13}C]isobutyrate		
	wt	*mutB*::*hyg*	*icmA*::*hyg*	wt	*mutB*::*hyg*	*icmA*::*hyg*
Acetate unit						
C-7	1.4	1.5	1.3	1.7	1.6	ND[a]
C-9	1.5	1.6	1.6	2.1	1.8	
C-13	1.5	1.3	1.4	2.8	1.7	
C-19	1.8	1.8	1.5	3.4	2.2	
C-25	1.6	1.6	1.3	2.1	1.8	
Propionate unit						
C-1	5.0	8.8	3.1	6.9	9.2	ND
C-3	5.1	8.8	3.0	7.7	9.3	
C-5	5.2	9.2	3.1	7.2	9.3	
C-11	4.6	8.8	3.1	8.2	8.6	
C-17	4.7	8.7	3.1	6.8	9.3	
C-21	5.3	9.5	3.2	7.1	9.4	
C-23	4.9	8.9	3.1	7.1	9.0	
Butyrate unit						
C-15	16.8	12.3	8.9	17.0	12.4	ND

[a] ND, not determined (see text).

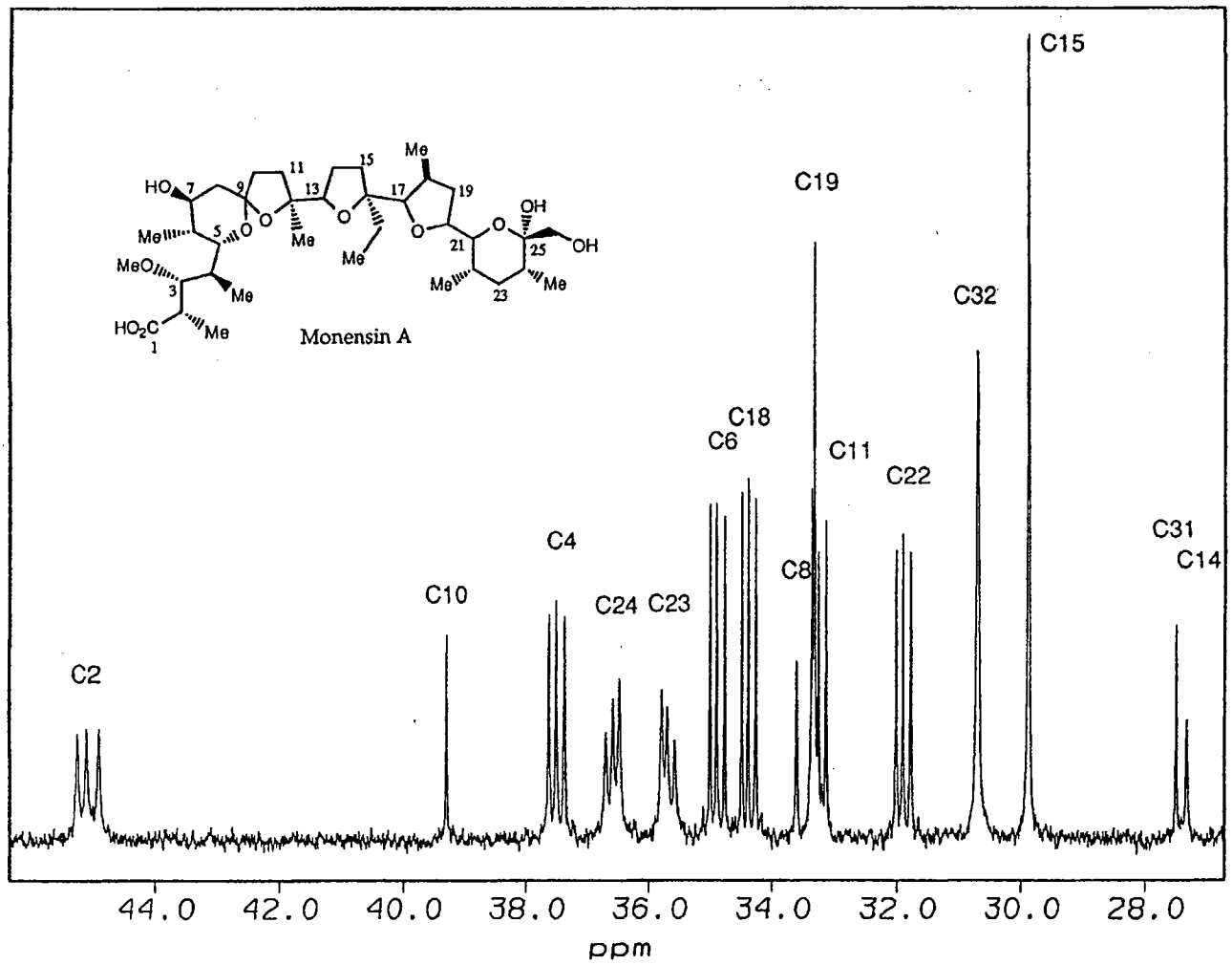

FIG. 2. A portion of the $^{13}C\{^1H\}$ NMR spectrum of monensin A isolated after feeding [1,3-$^{13}C_2$]ethyl acetoacetate to a *mutB*::*hygB* mutant of *S. cinnamonensis*. The strongly enriched signals from the *n*-butyrate unit in the backbone of monensin (at positions 15 and 32) are shown, along with the enriched doublets (for C-2, C-4, C-24, C-23, C-6, C-18, C-11, and C-22) surrounding the natural abundance signals for positions derived from propionate building blocks.

subunit (IcmB) of 136 residues (21). The MCM from this organism is a heterodimer with a large subunit (MutB) of 733 residues and a small subunit (MutA) of 616 residues (2). In this work, MCM was inactivated in *S. cinnamonensis* by inserting a *hygB* gene into *mutB*, to produce an *mutB*::*hygB* mutant. The cloning and insertional inactivation of *icmA* from *S. cinnamonensis* were reported earlier (33).

The effect of the *icmA* and *mutB* mutations on the growth of *S. cinnamonensis* was studied on solid minimal medium with various carbon sources. Both mutants show comparable growth on control plates containing glucose and succinate (Table 2). As with a previously reported knockout of MCM in *Nocardia corallina* (30), the *S. cinnamonensis mutB*::*hygB* mutant was unable to grow on propionate and valerate, since the conversion of methylmalonyl-CoA to succinyl-CoA was blocked (Fig. 1). The growth of this mutant was also strongly retarded on *n*- and isobutyrate, *n*- and isocaproate, and acetoacetate and crotonic acid as sole carbon sources.

The *icmA*::*hygB* mutant was unable to grow on valine, isobutyrate, or isocaproate, although it grew normally on acetate, butyrate, and valerate (Table 2). Moreover, addition of isobutyrate during growth in rich medium in liquid culture caused cessation of further growth and monensin production (see labeling studies in Materials and Methods and Results). The ability of both *mutB*::*hygB* and *icmA*::*hygB* mutants to grow normally on acetate as sole carbon source is inconsistent with the operation of a recently proposed (7) novel anaplerotic pathway that is an alternative to the glyoxylate cycle, for utilization of C_2 and C_4 fatty acids in streptomycetes.

Valine catabolism in pseudomonads and mammals (10, 15, 32) proceeds via isobutyryl-CoA, 3-hydroxybutyric acid, and methylmalonic acid semialdehyde, to propionyl-CoA (indicated in the dotted box in Fig. 1). The gene encoding methylmalonic acid semialdehyde dehydrogenase was cloned recently from *Streptomyces coelicolor* (34). An *msdA*::*hygA* mutant lost methylmalonic acid semialdehyde dehydrogenase activity as well as the ability to grow in a minimal medium with valine or isobutyrate as the sole carbon source. Nevertheless, labeling studies of many (but not all [19]) polyketide antibiotic-producing streptomycetes (3, 18, 20, 22, 24) have shown that [2-^{13}C]valine and [1-^{13}C]isobutyrate label C-1 of each propionate-derived subunit in the polyketide backbone (as indicated for monensin A in Fig. 1), rather than being lost as carbon dioxide. The results of these labeling experiments are incon-

sistent with a major flux through the *Pseudomonas*-mammalian pathway of valine catabolism during polyketide biosynthesis in the stationary phase in these streptomycetes, including *S. cinnamonensis*. Moreover, since the *S. cinnamonensis icmA::hygB* mutant was unable to grow on valine or isobutyrate, this *Pseudomonas*-mammalian pathway of valine catabolism is either absent or poorly expressed in this mutant during growth on minimal medium.

The conversion of isobutyryl-CoA to methylmalonyl-CoA by path A (Fig. 1) has been frequently discussed in studies of antibiotic-producing streptomycetes (29), including *S. cinnamonensis* (22). If path A is operative, however, this should provide a route for the utilization of valine and isobutyrate in the *icmA::hygB* mutant, and yet the mutant could not grow in minimal medium with isobutyrate or valine as sole carbon source (Table 2). It seems possible that, during antibiotic production in the stationary phase in a rich complex medium (28), a different pattern of gene expression and metabolite utilization may occur, compared to the situation on minimal agar. The inability of the *icmA::hygB* mutant to grow on isobutyrate and valine in minimal medium, therefore, may not rule out a role for path A (Fig. 1) in complex medium during the stationary phase in *S. cinnamonensis*.

An alternative route to methylmalonyl-CoA via succinyl-CoA might conceivably involve the conversion of isobutyryl-CoA to *n*-butyryl-CoA, with ω oxidation of the latter to succinyl-CoA (path B in Fig. 1). The reversible conversion of crotonyl-CoA to 4-hydroxybutyryl-CoA catalyzed by an oxygen-sensitive, flavin-containing enzyme is known for clostridia (23, 26), but it is so far unclear whether a similar enzyme occurs in streptomycetes, and no other route for the ω functionalization of *n*-butyryl-CoA is presently known. In addition, it was shown earlier (27) that trideuteriolabeled acetate can be efficiently incorporated into all the propionate units in monensin A without loss of deuterium. This observation rules out a major flux from *n*-butyryl-CoA to methylmalonyl-CoA via succinyl-CoA but is consistent with path A (Fig. 1). It should be noted, however, that similar experiments carried out with *Streptomyces longisporoflavus*, the producer of the polyether ICI139603 (also called tetronasin), showed that the C-methyl groups retain at most two deuterium atoms from acetate (5), as would be expected for an incorporation of the label via succinate and MCM (Fig. 1).

To determine how *n*- and isobutyrate are utilized for polyketide biosynthesis in the *icmA* and *mutB* mutant strains, ^{13}C-labeled precursors were fed to each, and the pattern of incorporation into monensin A was determined by ^{13}C NMR. An efficient incorporation of *n*-[1-^{13}C]butyrate into the propionate-derived units in the backbone of monensin A was observed in the wt and was higher in the *mutB* mutant but slightly lower in the *icmA* mutant (Table 3). The enrichments in acetate-derived units in monensin A were low, indicating a minimal breakdown of [^{13}C]butyrate to [^{13}C]acetate prior to incorporation. These results are consistent with a major flux through path A (Fig. 1) but show also that label is still incorporated at a lower efficiency, by an alternative route, when ICM is inactivated.

The efficient incorporation of [1-^{13}C]isobutyrate into the propionate units in monensin A in the *mutB::hygB* strain shows that the conversion of isobutyryl-CoA to methylmalonyl-CoA does not require MCM. Unfortunately, it was not possible to incorporate [1-^{13}C]isobutyrate into monensin in the *icmA* mutant, because addition of isobutyrate (or valine) to the culture arrested further growth and monensin production.

Finally, a labeling experiment with ethyl [1,3-$^{13}C_2$]acetoacetate was carried out to establish whether the incorporation of a C_4 precursor into the propionate units is possible without prior cleavage to acetate units. Most likely, the precursor is hydrolyzed by endogenous lipases or esterases, is activated in the cytoplasm to [1,3-$^{13}C_2$]acetoacetyl-CoA, and then is converted to *n*-butyryl-CoA by steps similar to those involved in fatty acid metabolism (31) or polyhydroxybutyrate metabolism (1). The monensin A isolated from both mutants and the wt showed an essentially identical pattern of one-bond ^{13}C-^{13}C couplings (Fig. 2), within each propionate unit, consistent with the formation and incorporation of [1,2-$^{13}C_2$]methylmalonyl-CoA without fragmentation of the precursor to acetate units. Hence, [1,3-$^{13}C_2$]acetoacetyl-CoA can be converted efficiently into [1,2-$^{13}C_2$]methylmalonyl-CoA in the absence of either ICM or MCM. Presently, it is uncertain how butyryl-CoA is converted intact into methylmalonyl-CoA when ICM is absent, although we disfavor path B in Fig. 1 for reasons discussed above.

At the present time, we are unable to identify one unifying metabolic plan that fully explains all the data discussed above. A scenario, however, in which a still unrecognized route allows the conversion of *n*-butyryl-CoA (or acetoacetyl-CoA) into methylmalonyl-CoA in streptomycetes may be considered. In this respect, it is interesting to note that a third MCM-like protein of unknown function called MEA has been described recently (7). MEA has been implicated in the assimilation of C_2 compounds in *Streptomyces collinus* and in methanol and ethanol utilization in *Methylobacterium extorquens* (25). Further clarification of C_2 and C_4 metabolism in streptomycetes may benefit from the elucidation of the function of this MEA protein.

ACKNOWLEDGMENTS

This work was supported financially by the Canton of Zurich.

J.W.V. and K.Z.-B. contributed equally to this work.

Grateful acknowledgment is made to Annelies Meier for expert technical assistance.

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